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News & Views

Mitochondrial Complex I Inhibition in Parkinson's Disease: How Can Curcumin Protect Mitochondria?

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ABSTRACT

Selective damage of mitochondrial complex I within the dopaminergic neurons of the substantia nigra is the central event during Parkinson disease. Peroxynitrite is one of the important free radicals probably mediating complex I damage. Peroxynitrite inhibits brain complex I mainly by 3-nitrotyrosine and nitrosothiol formation, but how these modifications alter the structure–function relation of complex I is unclear. Curcumin pretreatment protects brain mitochondria against peroxynitrite *in vitro* by direct detoxification and prevention of 3-nitrotyrosine formation and *in vivo* by elevation of total cellular glutathione levels. These results suggest a potential therapeutic role for curcumin against nitrosative stress in neurological disorders. *Antioxid. Redox Signal.* 9, 399–408.

SELECTIVE INHIBITION OF MITOCHONDRIAL COMPLEX I (NADH DEHYDROGENASE; CI) DURING PARKINSON DISEASE

Parkinson's disease (PD) is a neurodegenerative disease characterized by gradual loss of dopaminergic neurons within the substantia nigra (SN) (10). Oxidative/nitrosative stress and mitochondrial dysfunction are major events that occur during neuronal death in PD (1). It has been observed that the SN of early PD patients has a significant depletion of total glutathione (GSH+ GSSG). Glutathione depletion in the brain probably promotes mitochondrial damage via increased reactive oxygen species (ROS) (3). Previously, it was demonstrated that glutathione depletion in dopaminergic cells causes oxidative stress, leading to mitochondrial dysfunction (13).

During PD, a selective inhibition of CI results in mitochondrial dysfunction and neuronal loss (22). PD toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone act on dopaminergic neurons via selective CI inhibition. Several studies indicate that the selective CI inhibition during PD involves specific oxidative/nitrosative modifications of different CI subunits (13–15, 17, 18, 24). CI activity is considered to be among the most severely affected by age-related oxidative stress, resulting in mitochondrial dysfunction (15).

In our previous work, we showed that glutathione depletion mediated oxidative stress and/or increased nitrosative stress causes CI inhibition (2).

PEROXYNITRITE, A MAJOR REACTIVE NITROGEN SPECIES, DAMAGES CI

Peroxynitrite (PN), a short-lived RNS generated within mitochondria, is highly reactive and rapidly damages proteins by modifying tyrosines (nitration), cysteines (S-nitrosation), or tryptophans (*N*-formyl kynurenine). PN-mediated 3-nitrotyrosine (3-NT) modification may be significant during age-related neurodegeneration. Recent data show that intrastriatal injections of 3-NT into rat brain cause apoptosis of

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dopaminergic neurons (4). Accumulation of 3-NT–modified proteins has been observed in both PD and Alzheimer's disease (AD) (20, 23).

Mitochondria are particularly vulnerable to PN-mediated nitrosative stress. Prolonged exposure to nitric oxide (NO) inhibits cell respiration, possibly via PN-mediated CI inhibition due to S-nitrosation of critical thiols (7). Murray *et al*. (18) showed a direct correlation between PN-mediated CI inhibition and the 3-NT signature in CI subunits. Earlier, we detected in CI-enriched fractions from glutathione-depleted dopaminergic neurons, two bands with anti-3-NT immunoreactivity, indicating PN-dependent 3-NT formation during glutathione depletion-mediated CI inhibition (2). These studies emphasize the role of PN-mediated nitrocysteine and 3-NT formation in mitochondrial dysfunction during PD.

As a proof of principle, in this study, we incubated mouse brain mitochondria with increasing PN (250, 500, 750, and 1,000 μ*M*) followed by CI assay. Around 40% inhibition of CI was achieved at 750 μ*M* PN, consistent with previous reports (2, 18) (Fig. 1).

WHAT IS THE MECHANISM OF PN-MEDIATED CI INHIBITION?

We hypothesize that PN-mediated CI inhibition might occur through irreversible 3-NT modification or by reversible nitrosation (2, 11, 18). Evidently, PN-dependent CI inhibition was accompanied by a dose-dependent increase in 3-NT–modified mitochondrial proteins (Fig. 1C). Both CI inhibition and 3-NT formation were abolished by pretreatment with dithiothreitol (DTT) or glutathione (GSH) (both at 1 m*M*) (Fig. 2A and B), suggesting that thiol antioxidants protect CI by direct PN detoxification.

Post-PN treatment of mitochondria with DTT/GSH also restored CI activity significantly, albeit to a lesser extent than pretreatment. But post-treatment did not abolish the 3-NT signal (Fig. 2B), indicating that restoration of CI activity occurred probably by the reversal of nitrosation events. Other studies have suggested that CI can undergo specific nitrosation events (5). PN-treated mitochondria exhibited elevation in both free

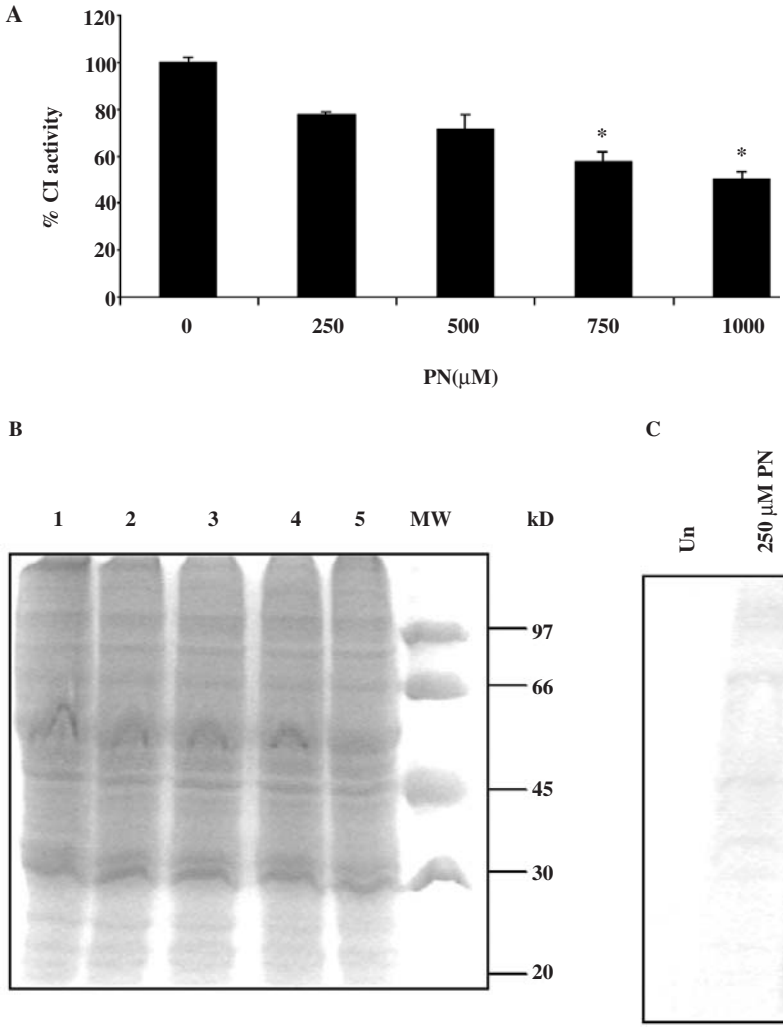

FIG. 1. Complex I activity and anti-3-nitrotyrosine Western blots in peroxynitrite-treated brain mitochondria. (**A**) Peroxynitrite (PN) (0–1,000 μ*M*) inhibits complex I (CI) activity in mouse brain mitochondria. Values shown as percentage of activity compared with 0 μ*M* PN (100% CI activity = 145 ± 3.5 nmol/min/mg of mitochondrial protein). **p* < 0.01 compared with untreated control. (**B**) Coomassie-stained gel of samples used for Western blot as in **C**. (**C**) Anti-3-NT Western blot of total proteins (35 μg/lane) from ±PN-treated brain mitochondria. Un, untreated mitochondria; MW, molecular weight marker.

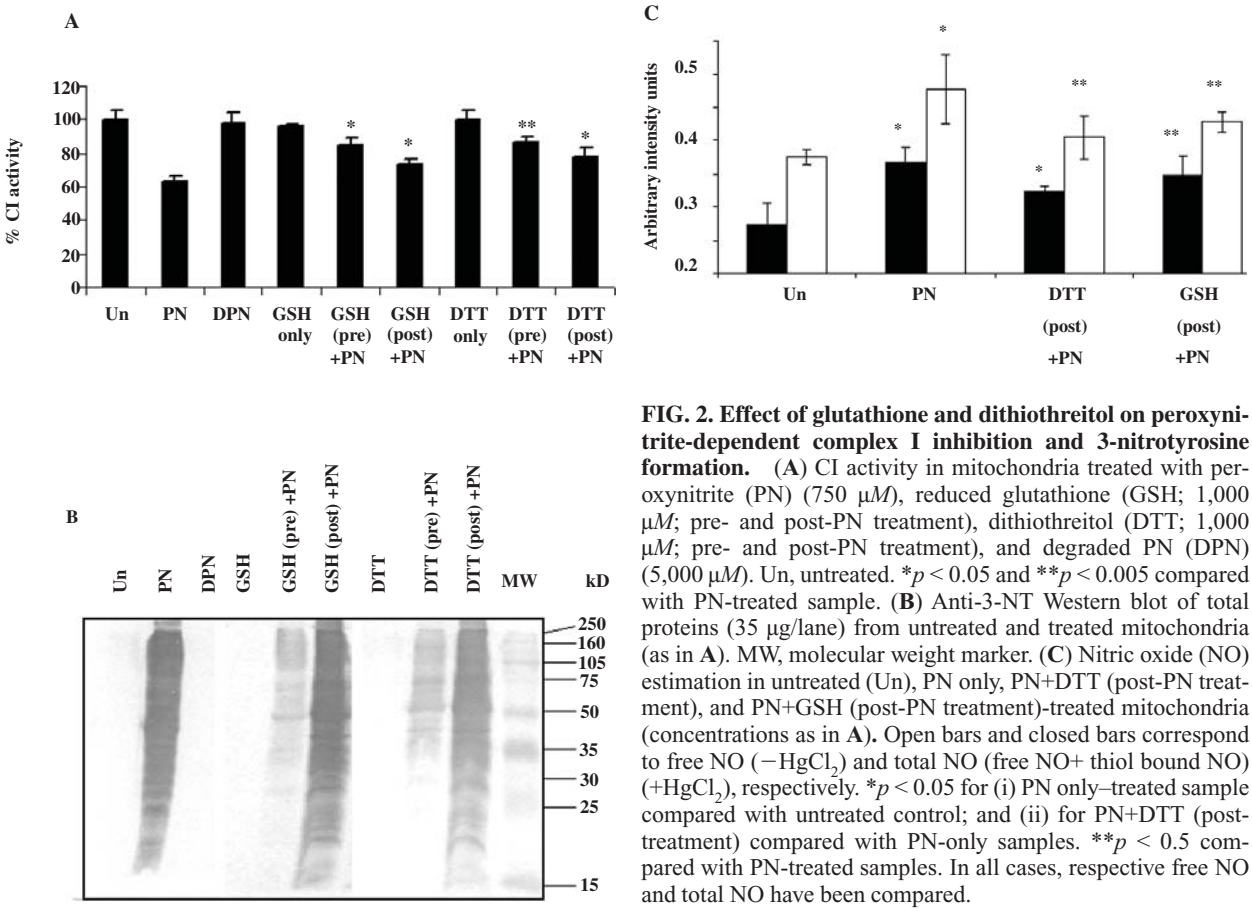

FIG. 2. Effect of glutathione and dithiothreitol on peroxynitrite-dependent complex I inhibition and 3-nitrotyrosine formation. (**A**) CI activity in mitochondria treated with peroxynitrite (PN) (750 μ*M*), reduced glutathione (GSH; 1,000 μ*M*; pre- and post-PN treatment), dithiothreitol (DTT; 1,000 μ*M*; pre- and post-PN treatment), and degraded PN (DPN) (5,000 μ*M*). Un, untreated. *$p < 0.05$ and **$p < 0.005$ compared with PN-treated sample. (**B**) Anti-3-NT Western blot of total proteins (35 μg/lane) from untreated and treated mitochondria (as in **A**). MW, molecular weight marker. (**C**) Nitric oxide (NO) estimation in untreated (Un), PN only, PN+DTT (post-PN treatment), and PN+GSH (post-PN treatment)-treated mitochondria (concentrations as in **A**)**.** Open bars and closed bars correspond to free NO ($-HgCl_2$) and total NO (free NO+ thiol bound NO) ($+HgCl_2$), respectively. *$p < 0.05$ for (i) PN only–treated sample compared with untreated control; and (ii) for PN+DTT (post-treatment) compared with PN-only samples. **$p < 0.5$ compared with PN-treated samples. In all cases, respective free NO and total NO have been compared.

NO and S-NO content, which was decreased significantly by DTT and GSH post-treatments (Fig. 2C). Earlier it was demonstrated that glutathione depletion in dopaminergic cells caused nitrosation-dependent selective CI inhibition (11).

CURCUMIN PROTECTS CI AGAINST PN TOXICITY *IN VITRO*

Because CI is highly vulnerable to nitrosative stress, antioxidants against PN-mediated mitochondrial toxicity during PD should be explored. Thiol reductants such as glutathione are not completely effective antioxidants *in vivo,* because of their decreased ability to cross the blood–brain barrier, although membrane-permeable glutathione esters have been used to enhance its bioavailability (3). Nevertheless, a demand exists for natural antioxidants that could easily cross the blood–brain barrier and also are nontoxic at high doses.

Curcumin is an active yellow phenolic compound. It is isolated from turmeric (*Curcuma longa* L.), a spice used in traditional Indian cuisine and medicine. Curcumin has been demonstrated to be an antioxidant and a potent anticancer agent (9). Recently, the potential of curcumin therapy in neurological disorders such as AD (16, 28) and stroke (26) was recognized. Although studies have used curcumin therapy in PD (29), its protection against PN toxicity has not been explored.

We have tested in our *in vitro* system two natural polyphenols, curcumin and resveratrol (Fig. 3A and E), for their ability to protect CI against PN-mediated mitochondrial toxicity. Curcumin (0–100 μ*M*) by itself did not inhibit CI activity (Fig. 3F, inset). Curcumin pretreatment (50 μ*M*) of PN-treated mitochondria significantly restored CI activity, concomitant with a decreased anti-3-NT signal (Fig. 3B and C). On the contrary, resveratrol, which shares the antioxidant and protective properties of curcumin, failed to protect CI against PN (Fig. 3F). This is because resveratrol inhibited CI independent of PN (Fig. 3F, inset) (30). However, resveratrol protected against 3-NT modification at lower concentrations (25 μ*M*) owing to more phenoxyl groups (three as opposed to two in curcumin) (Fig. 3G). These data suggest that curcumin specifically protects against PN-mediated CI inhibition and 3-NT formation. Post-PN treatment with curcumin (50 μ*M*) did not protect CI significantly, probably because of the inability of curcumin to prevent nitrosation (Fig. 3B and D).

To confirm that curcumin indeed protects CI activity by abolishing 3-NT formation, we partially purified CI from mouse brain mitochondria treated with (a) 750 μ*M* PN and (b) 750 μ*M* PN + 50 μ*M* curcumin (pre-PN treatment). Soluble mitochondrial extracts from both samples were fractionated by the sucrose gradient method (2). Fractions (1 ml) were collected, and CI-containing fractions were identified by anti-CI Western blots (Fig. 4A). In both samples, the CI-positive

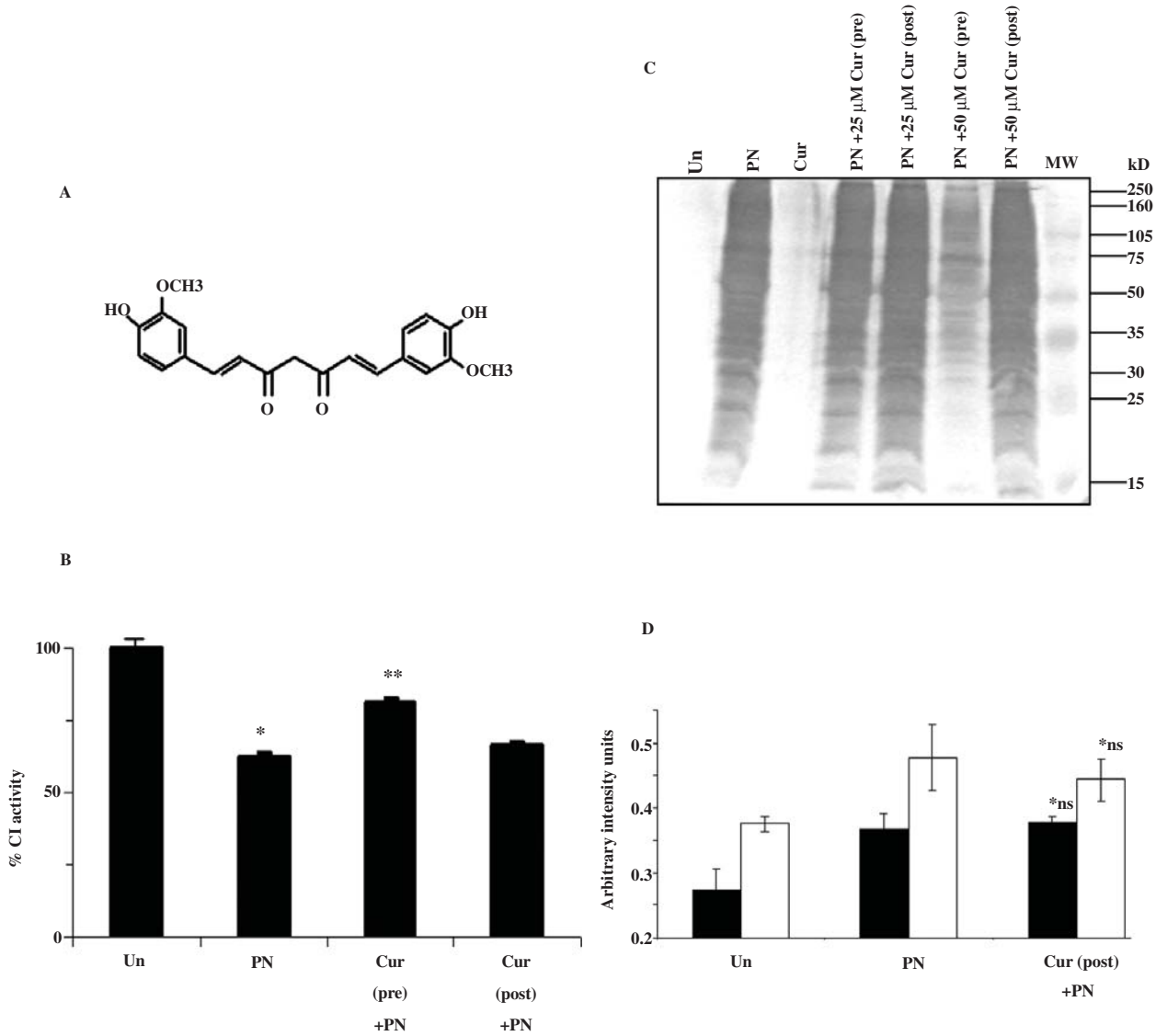

FIG. 3. Curcumin protects brain mitochondria against peroxynitrite-mediated complex I inhibition and 3-nitrotyrosine formation compared with resveratrol. (**A**) Structure of curcumin. (**B**) Percentage of complex I (CI) activity in brain mitochondria treated with peroxynitrite (PN) (750 μ*M*) alone, PN + 50 μ*M* curcumin (Cur; pre- and post-PN treatment). *$p < 0.001$ compared with untreated sample; **$p < 0.001$ compared with PN-treated sample. (**C**) Anti-3-NT Western blot of total proteins (35 μg/lane) from brain mitochondria treated with 750 μ*M* PN, PN + 25 μ*M* curcumin (Cur) (pre- and post-PN treatment), and PN + 50 m*M* curcumin (Cur) (pre- and post-PN treatment). MW, Molecular weight marker; Un, untreated control. (**D**) NO estimation in untreated (Un), PN-treated, and PN + curcumin (Cur) (post-treatment)-treated mitochondria (concentrations as in B). Open bars and closed bars correspond to free NO ($-HgCl_2$) and total NO (free NO + thiol-bound NO) ($+ HgCl_2$), respectively. *ns, not statistically significant compared with PN samples. In both cases, respective free NO and total NO were compared.

fractions showing maximum yield (fractions 8 and 9) were analyzed by blue native (BN) polyacrylamide gel electrophoresis (PAGE) to ascertain the intactness of CI (Fig. 4B). BN PAGE showed all mitochondrial complexes with intact CI migrating at ~700 kDa in all fractions (2, 21).

Curcumin pretreatment abolished PN-dependent nitration in CI-rich fractions compared with the PN control. This is shown by loss of anti-3-NT signal in sucrose fraction 8 in proteins <75 kDa (which corresponds to the largest subunit of CI). This suggests that curcumin protects CI by detoxifying PN and preventing 3-NT modification (Fig. 4D).

CURCUMIN AS A NEUROPROTECTIVE MOLECULE *IN VIVO* WITH IMPLICATIONS FOR PD THERAPY

Next, we incubated N27 dopaminergic neurons in culture with curcumin. Interestingly, curcumin induced a significant elevation of total cellular glutathione levels (Fig. 5A), thus providing increased protection against nitrosative stress. Curcumin increases glutathione levels via induced expression of glutamate-cysteine ligase, the rate-limiting enzyme in glu-

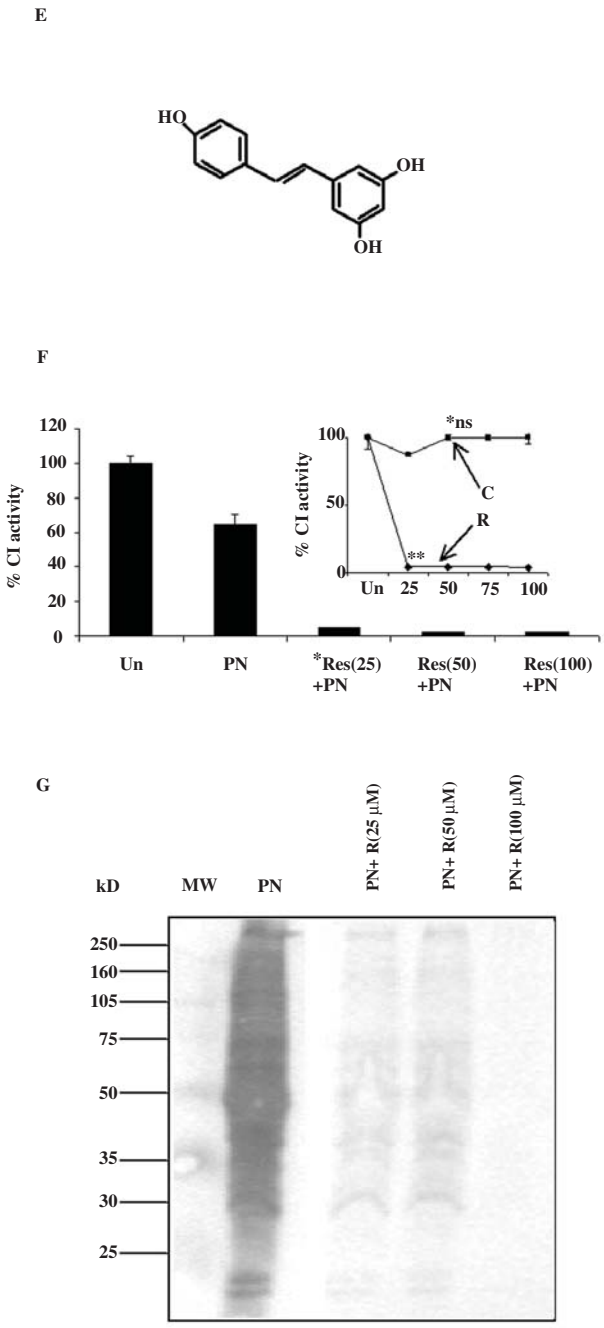

FIG. 3. Continued. (**E**) Structure of resveratrol. (**F**) Effect of resveratrol (R) (25, 50, and 100 μ*M*, as indicated in parentheses) pretreatment on complex I (CI) activity in untreated (Un) and peroxynitrite (PN) (750 μ*M*)-treated brain mitochondria. *$p < 0.05$ compared with PN sample. **Inset:** Percentage of complex I (CI) activity in resveratrol-only (R) and curcumin-only (**C**) treated mitochondria (both at μ*M* concentrations as indicated on x-axis). **$p < 0.001$ compared with untreated (Un) control. *ns (not significant) compared with untreated control. (**G**) Anti-3-NT Western blot (35 μg/lane) of total mitochondrial proteins after various treatments. Lanes: MW, Molecular weight marker; 1, PN (750 μ*M*); 2–5, PN + pretreatment with 25 μ*M*, 50 μ*M*, and 100 μ*M* resveratrol (R).

tathione synthesis (8). Mitochondria isolated from N27 cells incubated with 0.5 μ*M* curcumin exhibited CI protection against PN (Fig. 5B). We presume that an increased glutathione level probably protects mitochondria against PN (see Fig. 2A).

Similarly, intraperitoneal injections of curcumin into mice also caused a significant elevation of total glutathione levels in the brain (Fig. 5C). Total brain mitochondria isolated from curcumin-injected mice showed protection of CI against PN toxicity (Fig. 5D). These results suggest that curcumin protects against PN *in vitro* by direct detoxification, whereas it protects CI *in vivo* indirectly by elevation in cellular glutathione levels, which in turn detoxifies PN.

CONCLUSIONS AND OPEN QUESTIONS

The present study shows that PN could inhibit CI, a phenomenon significantly reversed by curcumin treatment. Initial experiments done as a proof of principle showed a correlation between PN-mediated CI inhibition and 3-NT formation/nitrosation (see Figs. 1 and 2). CI is inhibited only at high PN levels because of its short half-life at physiologic pH. PN at <250 μ*M* caused 3-NT modification of mitochondrial proteins (data not shown), but only around 500–1,000 μ*M*, we observed a direct correlation between PN-mediated CI inhibition and 3-NT formation (see Figs. 1A and 4D). Although such PN amounts might not accumulate *in vivo*, probably constant PN exposure for longer periods might cause protein damage. Exposure to 250 μ*M* PN for 5 s is probably equivalent to 7-min exposure to a steady-state PN at 1 μ*M* (19). These concentrations could be readily produced within mitochondria during PD, thus validating the *in vivo* relevance of our experiment. In our previous study (2), we depleted total glutathione in dopaminergic cells in the range relevant to PD. This caused limited nitration of CI subunits, suggesting that glutathione depletion caused constant exposure to PN, probably in the pathophysiologic range relevant to CI nitration.

Pretreatment with thiol antioxidants DTT and GSH protected mitochondria by direct PN detoxification (see Fig. 2A and B). We presume that the –SH groups in GSH and DTT might directly interact with PN, thus forming their respective S-NO derivatives. Post-treatment also restored CI activity, suggesting reversible nitrosation events in CI (see Fig. 2A). DTT and GSH might act on PN-mediated S-NO groups in CI, reducing them to SH. Figure 2C showed a direct correlation between CI recovery and NO/S-NO removal. It is also possible that both DTT and GSH bind to free NO, thus depleting the NO available for nitrosation. We propose that PN-dependent nitrosation is not as dramatic as 3-NT formation in mitochondria, as is evident from CI recovery and 3-NT Western blots compared with NO estimations (see Fig. 2). Nevertheless, nitrosation of mitochondrial thiols caused a small but significant and reversible effect on CI activity.

PN-mediated nitration or nitrosation probably disrupts the structure–function relation of CI, causing loss of activity. In a related study using postmortem human PD samples, we thoroughly analyzed all irreversible modifications in CI (unpublished data). PD samples showed higher levels of modification compared with controls. But how could such modifications affect CI activity? This could be answered only by

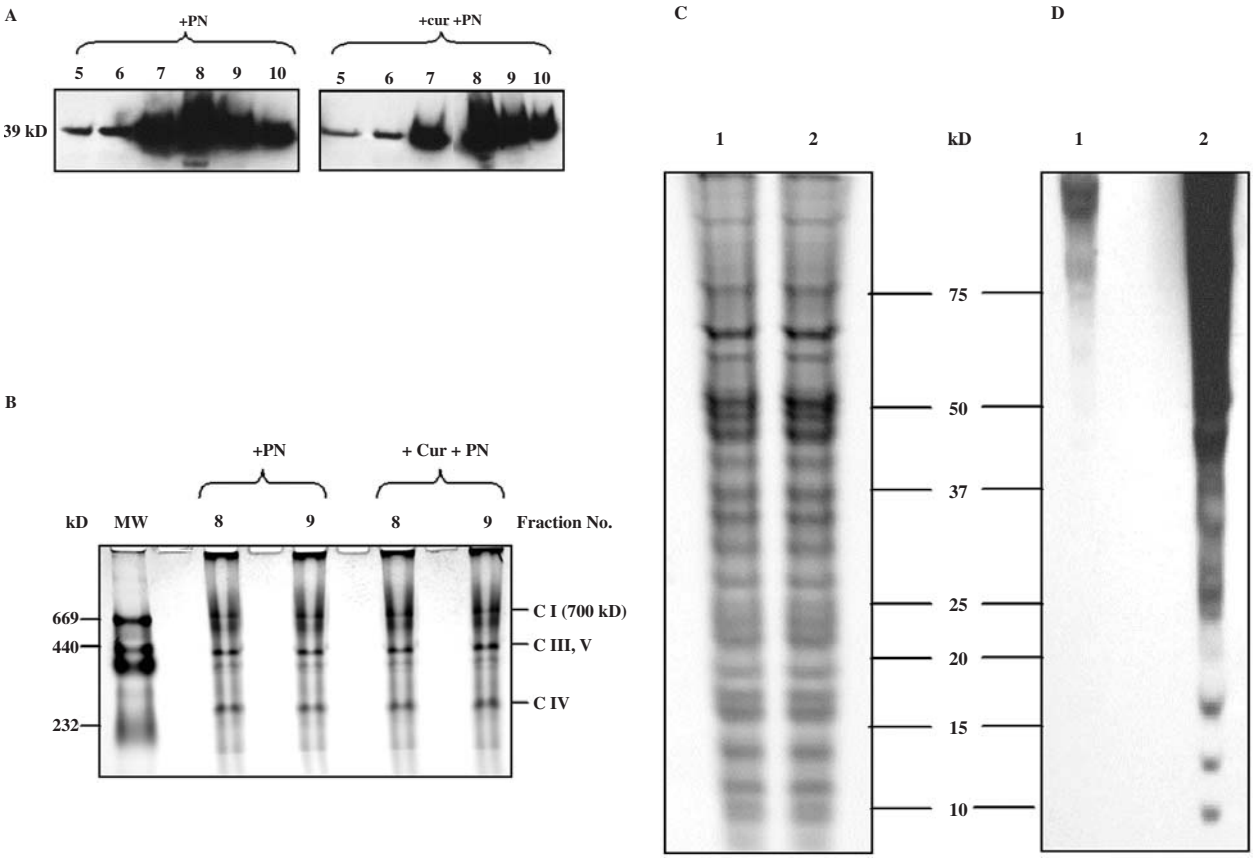

FIG. 4. Curcumin pretreatment protects neuronal complex I. (**A**) Brain mitochondria treated with 750 **μ***M* peroxynitrite only (PN) and 50 **μ***M* curcumin (cur) (pretreatment) + 750 **μ***M* PN were fractionated on sucrose gradient, and the fractions were analyzed for complex I (CI) by Western blots using anti-CI antibody against the 39-kDa subunit. Lanes 5–10 in both blots, corresponding to fractions 5 to 10 of the gradient, are CI-rich fractions. (**B**) Coomassie-stained 4–15% BN PAGE showing respiratory complexes in CI-rich sucrose gradient (as explained in **A**). MW, molecular weight marker; 8, 9, correspond to sucrose fractions 8 and 9 from PN-only and PN + curcumin samples. The bands corresponding to respiratory complexes I (CI, molecular mass, 700 kDa), III (CIII), IV (CIV), and V (CV) are highlighted on the right. (**C**) Sypro-ruby–stained SDS PAGE and (**D**) Anti-3-NT Western blot of sucrose fraction 8 (10 μg/lane). Lanes in both **C** and **D**: 1, PN + curcumin; 2, PN only (as explained in **A**).

carrying out x-ray analysis or theoretic molecular modeling of oxidative modifications in the 3D structure of CI.

Our data suggest that curcumin could be a potential neuroprotective agent in PD because of its anti-PN activity. Curcumin pretreatment protected CI against PN *in vitro* (see Fig. 3B) by direct detoxification, thus preventing 3-NT formation. Previous spectroscopic experiments showed that curcumin detoxifies PN via direct nitration of its phenoxyl groups (12). Interestingly, curcumin detoxified PN at a lower concentration (50 μ*M*) compared with DTT/GSH (1,000 μ*M*), indicating a more-potent antioxidant effect against PN. Curcumin might also protect by binding to CI and blocking PN attack, although no experimental evidence exists to prove this hypothesis.

The most important support for curcumin therapy in PD is based on the data indicating that curcumin induces brain glutathione synthesis *in vivo* (see Fig. 5A and C). We presume that elevated glutathione protects CI against PN toxicity (see Fig. 5B and D). Glutathione may prevent oxidative protein damage both by protecting protein thiol groups and by detoxifying PN or other free radicals. It is well known that glutathione depletion is one of the early events during PD pathology (3). Therefore, curcumin might induce elevation in glutathione levels *in vivo,* thus preventing oxidative overload on dopaminergic neurons. Curcumin therapy is advantageous because it easily crosses the blood–brain barrier and also is nontoxic at high concentrations. Curcumin therapy could be extended to other neurological disorders in which nitrosative stress plays an important role during pathology. It would be worthwhile to analyze the neuroprotective ability of curcumin against protein aggregation, proteasome inhibition, and microglial activation, making it a complete drug for PD therapy.

ABBREVIATIONS

BN, Blue Native; CI, mitochondrial complex I; DTT, dithiothreitol; GSH, glutathione (reduced); MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NO, nitric oxide; 3-NT, 3-nitrotyrosine; PAGE, polyacrylamide gel electrophoresis; PD, Parkinson disease; PN, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; S-NO, nitrosothiol; SN, substantia nigra.

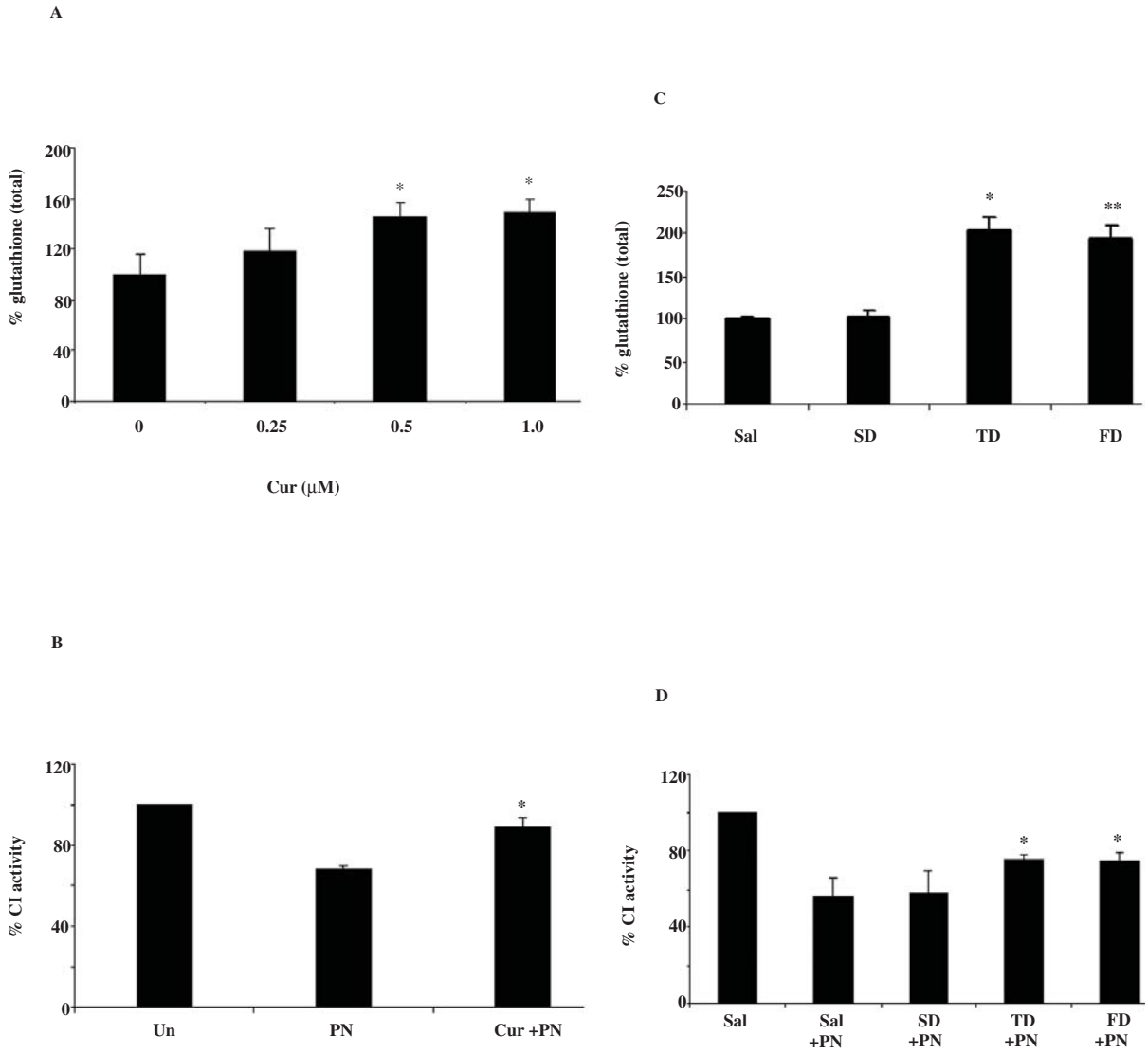

FIG. 5. Curcumin pretreatment protects neuronal complex I *in vivo*. (**A**) Effect of increasing concentrations of curcumin (Cur) (24 h) on total glutathione levels in N27 cells. Values shown as percentage glutathione compared with untreated control (100% total glutathione = 24 ± 4.0 nmol/mg protein). *$p < 0.05$ compared with untreated N27 cells. (**B**) Mitochondria isolated from N27 cells incubated with 0.5 μ*M* curcumin (24 h) (Cur) showed protection against 750 μ*M* peroxynitrite (PN), as shown by complex I (CI) activity. Values shown as percentage of activity compared with untreated (Un) control (100% CI activity = 7.4 ± 1.24 nmol/min/mg of mitochondrial protein). *$p < 0.005$ compared with CI activity in N27 mitochondria treated with PN only. (**C**) Total glutathione levels in whole brain extracts obtained from saline-injected controls (Sal) and curcumin-injected mice [50 mg/kg b.w. per day for 1 day = single dose (SD); 3 days = triple dose (TD); and 5 days = five doses (FD)]. Values shown as percentage of activity compared with Sal (100% glutathione = 1.6 ± 0.15 nmol/mg protein). *$p < 0.0005$ and **$p < 0.001$ compared with saline control. (**D**) Complex I (CI) activity was measured in brain mitochondria isolated from saline-injected controls (Sal) and curcumin-injected mice (50 mg/kg b.w. per day; SD, TD, and FD, respectively, as in **C**) followed by peroxynitrite treatment at 750 μ*M*. *$p < 0.005$ compared with PN-only treated mitochondria.

APPENDIX

Notes

1. Materials and Methods

All the chemicals and solvents were of analytic grade. Anti-CI mouse antibody against subcomplex subunit 9 (NDUFA9; 39 kDa) was obtained from Molecular Probes (Eugene, OR). Anti-3-NT antibody was obtained from Sigma (St. Louis, MO) and Molecular probes. All tissue-culture materials were procured from Sigma. SYPRO Ruby stain was obtained from Bio-Rad Laboratories (Hercules, CA). Nitrocellulose membrane was obtained from Millipore (Billerica, MA). Horseradish peroxidase conjugated secondary antibodies were obtained from Bangalore Genei (rabbit) and Chemicon, Millipore. PN was obtained from Upstate, Millipore. Protease inhibitor cocktail was procured from Sigma. N27

cell line was a kind gift from Dr. Curt Freed, University of Colorado (U.S.A.).

2. Cell line and tissue samples

Mouse brain and rat dopaminergic $1RB_3AN_{27}$ (N27) neuronal cells were used throughout this study. N27 cells were derived from embryonic rat mesencephalic neurons via SV40 large T antigen immortalization (2). N27 cells were grown in RPMI medium 1640 containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) and were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were subcultured once a week via trypsin treatment.

The N27 cell line was incubated with curcumin for 24 h followed by either preparation of soluble extracts for total glutathione estimation or mitochondrial preparations for PN treatment and CI assays.

3. Preparation, PN treatment, and extraction of mitochondria

Mitochondrial preparations were based on the method described earlier (2, 27). In brief, N27 cells were washed in buffer H (5 m*M* HEPES, 210 m*M* mannitol, 70 m*M* sucrose, 1 m*M* EGTA, and 0.5% bovine serum albumin) and resuspended in the same buffer. The cell suspension was homogenized and centrifuged at 800 *g* for 5 min at 4°C. The supernatant that was enriched in mitochondria was then centrifuged at 10,000 *g* for 20 min at 4°C. The resultant mitochondrial pellet was resuspended in buffer H and stored as aliquots at −80°C. For brain samples, freshly dissected tissue was washed and homogenized in ice-cold isolation buffer [320 m*M* sucrose, 5 m*M* TES [tris(hydroxymethyl)methylaminoethanesulfonic acid, 1 m*M* EGTA, pH 7.2]. The homogenate was centrifuged at 1,000 *g* for 5 min at 4°C, and then the supernatant was centrifuged at 8,500 *g* for 10 min at 4°C. The mitochondrial enriched pellet was resuspended in isolation buffer, layered on top of 6% (wt/vol) Ficoll solution, and centrifuged at 75,000 *g* for 30 min at 4°C to remove myelin, which forms a layer at the top. The pellet was resuspended in reconstitution buffer (250 m*M* sucrose, 10 m*M* TES, pH 7.2) and stored as aliquots at −80°C.

PN treatment of mitochondria was carried out as described earlier (2, 18). In brief, PN solution was placed on the wall of the tube containing mitochondria (suspended at 5 mg/ml protein concentration in 25 m*M* phosphate buffer pH 7.2 + 5 m*M* $MgCl_2$) and vortex-mixed for a few seconds to ensure proper mixing before degradation of PN. These mitochondria were used in CI assays and the sucrose-gradient purification protocol.

For preparation of extracts, ~2 mg of mitochondria was washed with 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and suspended in the same buffer containing protease inhibitor cocktail. This suspension was solubilized by adding *n*-dodecyl β-D-maltoside (Sigma) to a final concentration of 1% at 5 mg/ml protein concentration and incubated for 30 min on ice. Insoluble material from this suspension was removed by centrifugation at 16,000 *g* for 30 min at 4°C.

4. CI purification by sucrose gradient centrifugation

Sucrose-gradient experiments were performed as described earlier (2). Mitochondrial extract prepared as described was subjected to a 10–35% sucrose-step gradient consisting of 1-ml step fractions of 35, 32.5, 30, 27.5, 25, 22.5, 20, 17.5, 15, and 10% prepared in 10 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 0.05% maltoside. The mitochondrial sample was loaded onto the gradient in 5% sucrose and centrifuged at 38,000 rpm for 16.5 h at 4°C in an Optima-XL 100 K ultracentrifuge (SW40 Ti swinging-bucket rotor) from Beckman (Fullerton, CA). Fractions of 1 ml were collected from the top, frozen immediately, and stored at −80°C. Each fraction was concentrated to 100 µl by using a Microcon-100 concentrator (Millipore). An aliquot of 20 µl of each concentrated fraction was analyzed by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), followed by Western blotting with an anti-CI monoclonal antibody against the 39-kDa CI subunit NDUFA9 to ascertain fractions containing CI.

5. Blue Native (BN) PAGE

Sucrose density gradient fractions that were positive for CI, as indicated by Western analysis with the 39-kDa monoclonal antibody were pooled, concentrated, and subjected to BN PAGE analysis according to the method described earlier (2, 21). In brief, to 20 µl of concentrated pooled CI fractions (20% of total sample volume), 20 µl of BN sample buffer (1.5 *M* aminocaproic acid, 0.05 *M* Bis-Tris, pH 7.0), 2.5 µl of 10% *n*-dodecyl b-D-maltoside, 2.5 µl of protease inhibitor cocktail, and 4 µl of gel loading buffer (5% Serva Coomassie Brilliant Blue G-250, 1 *M* aminocaproic acid) were added and loaded onto a 4–15% BN Tris gel (Bio-Rad Laboratories). A mixture of 50 m*M* Tricine, 15 m*M* Bis-Tris, pH 7.0, and 0.02% Coomassie Blue was used as a cathode buffer and 50 m*M* Bis-Tris, pH 7.0, as an anode buffer. The gel was run at 4°C at 5 mA for the first 12 h and then at 10 mA until the dye marker reached the bottom of the gel. The gel was stained with 0.25% Coomassie Brilliant Blue G-250 (Serva Electrophoresis, Heidelberg, Germany) for 1 h, destained with 50% methanol/10% acetic acid for 1 h, and then destained with 10% methanol/10% acetic acid overnight.

6. SDS PAGE and Western analysis

Mitochondrial protein extracts (35 µg/lane) or CI-enriched fractions (~10 µg/lane) were run on 12% SDS PAGE at 100 V for ~2.5 h. Gels were stained either with Coomassie Brilliant Blue R-250 (Sigma) or SYPRO Ruby (Biorad). For SYPRO Ruby staining, SDS gels were fixed with 10% methanol, 7% acetic acid for 30 min, and subsequently stained with SYPRO Ruby, followed by destaining in 10% methanol/7% acetic acid.

For Western blot, proteins from SDS gels were electrophoretically transferred to nitrocellulose membranes in a semidry apparatus for 2 h at 125 mA. Nonspecific binding was blocked by incubating membranes in 1X PBS containing Tween-20 (Sigma) and 5% milk powder for 1 h at room temperature or overnight at 4°C. The membranes were incubated with primary antibodies in PBS/Tween containing 5% milk powder for 1.5 h at room temperature. Blots were washed with PBS/Tween and then incubated for 1.5 h at room temperature with HRP-conjugated secondary antibodies in PBS/Tween containing 5% milk powder. Membranes were washed with PBS/Tween, and the color reaction was developed in 1x PBS contain diamino benzidine at 1 mg/ml (wt/vol) (Sigma) and 0.1% H_2O_2.

7. CI enzyme assay

CI enzyme assays were carried out as described earlier (2, 27). In brief, the assay was initiated by addition of aliquots of mitochondria to 50 m*M* potassium phosphate/Tris-HCl, pH 7.4, 500 µ*M* EDTA, 1% bovine serum albumin, 200 µ*M* NADH, and 200 µ*M* decylubiquinone with and without 2 µ*M* rotenone in the presence of KCN with 0.002% dichloroindophenol as a secondary electron acceptor. The decrease in the absorbance at 600 nm was recorded as a measure of enzyme reaction rate at 37°C for 10 min, and specific activity was calculated. The results were plotted as relative rotenone-sensitive specific activity.

8. Griess Assay to detect NO and S-NO

NO estimations based on the Saville reaction were carried out as described by Chung *et al.* (6) with some modifications. In brief, treated mitochondria were centrifuged (10,000 *g*/10 min/4ºC) and resuspended in 1x PBS containing 10% glycerol, 4 m*M* EGTA, and protease inhibitors. The samples were incubated with neutral Griess reagent [57.8 m*M* sulfanilic acid and 7.7 m*M* *N*-(1-naphthyl) ethylenediamine (Sigma) in PBS] ± $HgCl_2$ (1 m*M* final concentration). The reaction mixture was gently mixed, incubated for 20 min at room tempera-

ture, and absorbance was measured at 496 nm. All estimations were done in triplicate and normalized per protein.

9. Total glutathione (GSH+GSSG) estimation

Soluble extracts from mouse brain, and N27 cells were subjected to total glutathione estimations by 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) recycling method, as described earlier (25). Brain tissue was homogenized in PE buffer (100 m*M* potassium phosphate buffer, pH 7.4, containing 1 m*M* EDTA), and total protein in the homogenate was estimated in triplicate. Eighty microliters of the homogenate was acid precipitated with an equal volume of 2% sulfosalicylic acid (wt/vol). The mixture was centrifuged at 12,000 rpm (15 min), and the resultant supernatant was used for glutathione estimations. A 20-µl aliquot of the supernatant was incubated with assay buffer [PE buffer containing 0.8 m*M* DTNB and 0.32 U/ml glutathione reductase (Sigma)] in a final reaction volume of 450 µl. The reaction was initiated by addition of 0.6 m*M* NADPH. The reaction kinetics of DTNB recycling, which was dependent on total glutathione levels, was monitored at 412 nm for 3 min. The glutathione amount in each sample was calculated based on the maximum reaction rate compared with GSSG standards (0–250 ng). For N27 cells, the protocol was same, except that 25-µl aliquots of homogenate were used, and GSSG standards were from 0 to 100 ng. All estimations were conducted in triplicate, normalized per protein.

10. Curcumin experiments in vivo

All animal experiments were carried out in accordance with NIMHANS Institute Guidelines for the Care and Use of Laboratory Animals. Adult male C57BL/6 mice (10-week-old) weighing ~25 g each (n = 3 for each treatment) were obtained from the Central Animal Research Facility (CARF), NIMHANS. Mice were housed five per cage with food and water *ad libitum* in a well-ventilated room, and all animals were exposed to 12-h light and dark cycles. Mice were intraperitoneally injected with curcumin at 50 mg/kg b.w. per day for 1, 3, and 5 days. Animals were killed by decapitation 24 h after the final injection at each time point. Brains were recovered and used for glutathione and CI assays. Control animals were housed under identical conditions and received saline only by the same route.

11. Statistical analyses

All quantitative data were accumulated from at least three independent experiments. The final data are expressed as mean ± SD. Differences between mean values were analyzed by one-way analysis of variance (ANOVA).

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